

The signal transducer and activator of transcription 6 gene (*STAT6*) increases the propensity of patients with atopic dermatitis toward disseminated viral skin infections

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with increased susceptibility to recurrent skin infections.

Objective: We sought to determine why a subset of patients with AD have an increased risk of disseminated viral skin infections.

Methods: Human subjects with AD with a history of eczema herpeticum (EH) and various control groups were enrolled. Vaccinia virus (VV) expression was measured by means of PCR and immunofluorescent staining in skin biopsy specimens from each study group after incubation with VV. Transgenic mice with a constitutively active signal transducer and activator of transcription 6 gene (*STAT6*) were characterized for response to VV skin inoculation. Genotyping for 10 *STAT6* single nucleotide polymorphisms (SNPs) was performed in a white patient sample (n = 444).

Results: VV gene and protein expression were significantly increased in the skin of patients with EH compared with other

subject groups after incubation with VV *in vitro*. Antibody neutralization of IL-4 and IL-13 resulted in lower VV replication in patients with a history of EH. Mice that expressed a constitutively active *STAT6* gene compared with wild-type mice had increased mortality and satellite lesion formation after VV skin inoculation. Significant associations were observed between *STAT6* SNPs and EH (rs3024975, rs841718, rs167769, and rs703817) and IFN- γ production. The strongest association was observed for a 2-SNP haplotype (patients with AD with a history of EH vs patients with AD without a history of EH, 24.9% vs 9.2%; $P = 5.17 \times 10^{-6}$).

Conclusion: The *STAT6* gene increases viral replication in the skin of patients with AD with a history of EH. Further genetic association studies and functional investigations are warranted. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Atopic dermatitis, *STAT6*, eczema, virus, infection

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Atopic dermatitis (AD) is a genetically complex, chronic inflammatory skin disease that affects approximately 20% of all children and often persists in adults.^{1,2} The skin of patients with AD is characterized by a defective skin barrier and increased T_H2 responses.³ Additionally, some patients with AD have recurrent bacterial and disseminated viral skin infections, such as eczema herpeticum (EH).⁴

Vaccinia virus (VV) was routinely used for vaccination against smallpox until the worldwide vaccination program ceased in the late 1970s after the global eradication of smallpox.⁵ Although the vaccine was effective at conferring protective immunity in the general public, vaccination was associated with increased numbers of severe adverse effects in a subset of patients with AD. The re-emergence of orthopoxviruses as a human health concern prompted health officials to reinstitute voluntary smallpox vaccination in first-line responders⁶; however, the Centers for Disease Control and Prevention recommended that patients with allergic skin disease refrain because of the potential for severe adverse events.⁷ On the basis of this recommendation, more than 30% of our military refrained from vaccination, posing a significant health concern.⁸

The National Institute of Allergy and Infectious Diseases established the Atopic Dermatitis Vaccinia Network (ADVNet) to further characterize the immune response in the skin of patients with AD after VV inoculation in the skin and to identify genetic

Abbreviations used

AD:	Atopic dermatitis
ADVN:	Atopic Dermatitis Vaccinia Network
ADEH+:	Atopic dermatitis with a history of eczema herpeticum
ADEH-:	Atopic dermatitis without a history of eczema herpeticum
CT:	2-SNP haplotype
EH:	Eczema herpeticum
EV:	Eczema vaccinatum
FLG:	Filaggrin
HSV:	Herpes simplex virus
LD:	Linkage disequilibrium
MFI:	Mean fluorescence intensity
OR:	Odds ratio
SNP:	Single nucleotide polymorphism
STAT:	Signal transducer and activator of transcription
tIgE:	Total serum IgE
VV:	Vaccinia virus

biomarkers for those patients with AD with an increased propensity toward disseminated viral skin infections, such as EH. In a previous study we reported that skin from patients with severe AD showed increased VV replication.⁹ More recently, we found that patients with AD with a history of EH (ADEH+ patients) had significantly higher serum IgE levels than patients with AD without a history of EH (ADEH- patients).¹⁰ Because IL-4 and IL-13, which play a key role in isotype switching to IgE synthesis, act through the signal transducer and activator of transcription 6 gene (*STAT6*), we speculated that abnormalities in *STAT6* function caused by genetic variation could be associated with ADEH+ and increased VV replication in the skin of such patients.

METHODS**Study subjects**

For initial studies of viral replication in the skin of ADEH+ patients versus those in the control group (Table I), skin biopsy specimens were obtained from 55 patients with active AD (ADEH-), 20 patients with AD and a history of EH (ADEH+), 38 asthmatic patients, 35 healthy subjects with no history of skin disease, 36 patients with psoriasis, and 36 patients with a history of AD (quiescent AD). The means $\pm$ SEMs for age, total serum IgE (tIgE) level, and number of positive RAST test results are summarized for each subject group in Table I. Table II describes the racial distribution among skin biopsy specimen donors. Additional ADEH- patients ($n = 17$), ADEH+ patients ($n = 6$), and healthy subjects ($n = 19$) were recruited to test the effect of T_H2 cytokines or neutralization of T_H2 cytokines on viral replication in the skin.

Genotyping was conducted on 278 unrelated white patients with AD (of whom 112 were ADEH+) and 166 healthy control subjects participating in the ADVN. Clinical characteristics for this sample have been described previously.¹¹

Patients had not received topical corticosteroids for a period of 1 week before enrollment or systemic immunosuppressives for 1 month before enrollment in this study. This study was approved by the Institutional Review Boards at National Jewish Health, Johns Hopkins Medical Institute, Oregon Health & Sciences University, the University of Rochester Medical Center, the University of California San Diego, and Children's Hospital Boston. All subjects provided written informed consent before participation in these studies.

Viral source and culture

The Wyeth/ACAM 2000 strain of VV was obtained from the Centers for Disease Control and Prevention (Atlanta, Ga). The Western Reserve VV strain 1354 was obtained from the American Type Culture Collection (Manassas, Va) for use in murine studies.

Human skin explant cultures

Two-millimeter punch biopsy specimens were collected from the nonlesional skin of each enrolled subject. Skin biopsy specimens were cultured in the presence of media alone (RPMI supplemented with 10% FCS) or 2.5×10^5 platelet-forming units of VV for 24 hours. After the exposure period, media were removed, and biopsy specimens were submerged in 10% buffered formalin for immunofluorescent staining or Tri-Reagent (Molecular Research Center, Inc, Cincinnati, Ohio) for DNA analysis.

In some experiments anti-human IL-4 mAb (1 μ g/mL; R&D Systems, Minneapolis, Min) and anti-human IL-13 mAb (1 μ g/mL, R&D Systems) were added to the skin biopsy specimens from ADEH- ($n = 20$) and ADEH+ ($n = 10$) patients for 24 hours to neutralize T_H2 cytokines before infection with VV. After the viral exposure, biopsy specimens were cultured for an additional 24 hours and prepared for DNA isolation to measure viral copies.

VV DNA analysis

DNA was prepared from human skin explants according to the manufacturer's guidelines (Molecular Research Center, Inc, Cincinnati, Ohio). Viral copies were quantified by means of real-time PCR with primers specific for vaccinia ribonucleotide reductase (Vv14L), as previously described.¹²

VV immunofluorescent staining

Paraffin-embedded tissues were cut at 5 mm on frosted microscope slides. By using toluene and a series of ethanol washes, slides were deparaffinized and then rehydrated. Skin sections were then blocked with 5% BSA in Super Block (ScyTek Laboratories, Logan, Utah) containing 10% nonimmune donkey serum (Jackson Laboratories, West Grove, Pa) for 60 minutes. Slides were then stained with a rabbit anti-A27 antibody (Abcam, Cambridge, Mass) directed against an early viral protein located in the nucleus and cytoplasm of infected cells or control rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) at 4°C overnight. Slides were washed with PBS/Tween 0.05%, followed by incubation with a Cy3-conjugated donkey anti-rabbit IgG (Jackson Laboratories, Bar Harbor, Me).

Immunohistochemical staining was visualized with confocal microscopy (Leica, Wetzlar Germany). Slides were coded to ensure patients' anonymity. Images were collected at $\times 40$ magnification, and levels of mean fluorescence intensity (MFI) were measured with Slidebook 4.1 (Intelligent Imaging innovations, Denver, Colo). MFI was determined for each exposure group and was reported as the mean $\pm$ SE.

Mice

STAT-6VT transgenic mice with constitutively active *STAT6* were generated at Indiana University, as previously described.¹³ Briefly, V547 and T548 of the human *STAT6* gene were mutated to alanines under the transcriptional control of the CD2 locus control region. C57Bl/6 mice were purchased from Jackson Laboratories to generate hemizygous STAT-6VT and wild-type littermate mice. All mice were maintained in specific pathogen-free conditions, and experiments were approved by the Indiana University and National Jewish Health Institutional Animal Care and Use Committees.

IFN- γ ELISPOT assay

The differential immune responses (*ex vivo*) to herpes simplex virus (HSV) have been investigated by measuring IFN- γ production in isolated PBMCs from a subset of the ADVN sample (64 subjects), and clinical characteristics of participants have been previously described.¹⁴ IFN- γ production was examined by using the enzyme-linked immunosorbent spot (ELISPOT) assay adapted from the protocol, as previously described.¹⁵

Genotyping and quality control

Because no common variants were detected in the coding region of *STAT6*, we selected 6 tagging single nucleotide polymorphisms (SNPs) and 4 SNPs consisting of the promoter SNP rs12368672, the 3'-untranslated region SNP

TABLE I. Characteristics of skin biopsy specimen donors

Subject group	Age (y), mean $\pm$ SEM	Serum IgE (KU/L), geometric mean $\pm$ SEM*	No. of IgE allergen-positive ImmunoCAP test results, mean $\pm$ SEM
ADEH [−] patients (n = 44)	32.6 $\pm$ 1.98	137.4 $\pm$ 51.97	5.2 $\pm$ 0.59
ADEH ⁺ patients (n = 18)	21.6 $\pm$ 4.14	536.4 $\pm$ 288.97	6.7 $\pm$ 1.06
Asthmatic patients (n = 30)	31.4 $\pm$ 1.99	77.05 $\pm$ 29.40	3.8 $\pm$ 0.60
Healthy subjects (n = 42)	29.2 $\pm$ 1.18	24.62 $\pm$ 7.19	2.0 $\pm$ 0.40
Patients with psoriasis (n = 29)	40.4 $\pm$ 2.48	28.21 $\pm$ 10.89	1.6 $\pm$ 0.45
Patients with quiescent AD (n = 28)	34.3 $\pm$ 2.15	54.50 $\pm$ 20.09	2.8 $\pm$ 0.53

*Back-transformed SEs were computed by using the delta method.

TABLE II. Racial distribution among skin biopsy specimen donors

Race	No.
Indian	5
Asian	5
White	166
Other	12
Multiracial	3

rs703817, and 2 intronic SNPs close to the initially identified region. These SNPs were distributed in a 22.6-kb region on chromosome 12q13 covering the *STAT6* gene. The strategy for the tagging SNP selection has been previously described.¹⁶ SNPs were genotyped with TaqMan Allelic Discrimination Assays on the 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif). Potential confounding caused by population substructure was assessed by using genotype data from the 74 ancestry informative markers, as described previously.¹¹

Statistical analyses

Statistical analysis of viral data was conducted with GraphPad Prism version 5.03 (GraphPad Software, Inc, San Diego, Calif) and SAS version 9.1 (SAS Institute, Inc, Cary, NC). Comparisons of quantitative variables (eg, viral replication) among diagnostic groups were made by using 1-way ANOVA, followed by *t* tests for pairwise comparisons. The Tukey-Kramer method was used to control the family-wise type I error rate at 0.05. Survival analysis comparisons across groups of mice were computed with log-rank tests. Significant differences were conferred at a *P* value of less than .05. Genetic association analysis was performed with the Cochran-Armitage trend test under an additive model by using PLINK software.¹⁷ Associations with the log-transformed tIgE level, the Eczema Area and Severity Index score, and log-transformed spot-forming units/10⁶ PBMCs were performed by using a linear regression analysis adjusted for confounding variables (age and sex). Means of log-transformed values were back-transformed (geometric means) and back-transformed SEs were estimated by using the delta method to report tIgE statistics. Haplotype analyses were performed with sliding windows of 2 to 5 SNPs in which empiric *P* values for haplotype frequency differences were generated over 10,000 permutations. Analyses of the effect of anti-T_H2 cytokines were conducted by using paired *t* tests (eg, effect of anti-T_H2 antibodies vs media control on viral replication). Our power calculation with the QUANTO version 1.1 program¹⁸ demonstrated that the study population provided sufficient power (80%) to detect a genotypic odds ratio (OR) of 2.06, even if the allele frequency with the disease is only 10%.

RESULTS

Increased viral replication in ADEH⁺ patients

Skin biopsy specimens were collected from all subject groups in Table I to compare the ability of VV to replicate *ex vivo* in ADEH⁺ skin versus the skin of various control groups. Biopsy

specimens were treated with the standard human dose of VV used in smallpox vaccinations (2×10^5 platelet-forming units). Fig 1, A, demonstrates that skin from ADEH⁺ patients supported significantly greater VV replication (mean, 2.42 ± 0.63 VV viral copies/ng total DNA) than skin from ADEH[−] patients (1.03 ± 0.20 VV viral copies/ng total DNA, *P* = .01), asthmatic patients (0.12 ± 0.03 VV viral copies/ng total DNA, *P* < .001), healthy subjects (0.34 ± 0.10 VV viral copies/ng total DNA, *P* < .001), patients with psoriasis (0.70 ± 0.16 VV viral copies/ng total DNA, *P* < .01), and patients with quiescent AD (0.15 ± 0.04 VV viral copies/ng total DNA, *P* < .001). Additionally, VV replication was significantly greater in ADEH[−] skin compared with that seen in skin from asthmatic patients (*P* < .001), healthy subjects (*P* < .01), and patients with quiescent AD (*P* < .01). Immunofluorescent staining for an early viral protein confirmed higher levels of VV replication in the skin of ADEH⁺ patients compared with that of the other subject groups (Fig 1, B and C).

ADEH⁺ patients are characterized by a significantly greater T_H2 response (ie, increased serum IgE level and increased number of positive RAST test results) than ADEH[−] patients.¹⁰ Because the T_H2 response is characterized by increased IL-4 and IL-13 levels, we preincubated skin biopsy specimens from ADEH[−] and ADEH⁺ patients with antibodies against IL-4 and IL-13 to determine whether neutralization of T_H2 cytokines would inhibit VV replication. Pretreating ADEH[−] skin biopsy specimens with neutralizing antibodies did not significantly inhibit VV replication (Fig 2, A). In contrast, VV replication was significantly less in ADEH⁺ skin biopsy specimens pretreated with neutralizing antibodies (0.0009 ± 0.0005 VV copies/ng DNA, *P* = .053) compared with that seen in control-treated skin biopsy specimens (0.0024 ± 0.0007 VV copies/ng DNA; Fig 2, B).

Viral responses in STAT-6VT mice

To determine whether constitutive activation of STAT-6 modulates the response to VV, we inoculated 4-week-old STAT-6VT and wild-type mice with 5×10^6 platelet-forming units of VV (WR1354). After infection, mice were monitored for death and the development of satellite lesions. At the time of inoculation, none of the mice had any areas of skin irritation or rash. Interestingly, increased morbidity and mortality were observed in VV-infected STAT-6VT mice. This was evidenced by satellite lesions in significantly more STAT-6VT mice on day 11 (50.0% of mice) compared with wild-type mice (14.3% of mice; *P* = .022, Fisher exact test; Fig 3, A). Satellite lesions are an indicator of systemic infection that can be fatal. Supporting this notion, we found higher rates of survival in wild-type mice compared with STAT-6VT mice, reaching significance on day 16 (wild-type mice, 68.5% survival; STAT-6VT mice, 10.0% survival; *P* < .001,

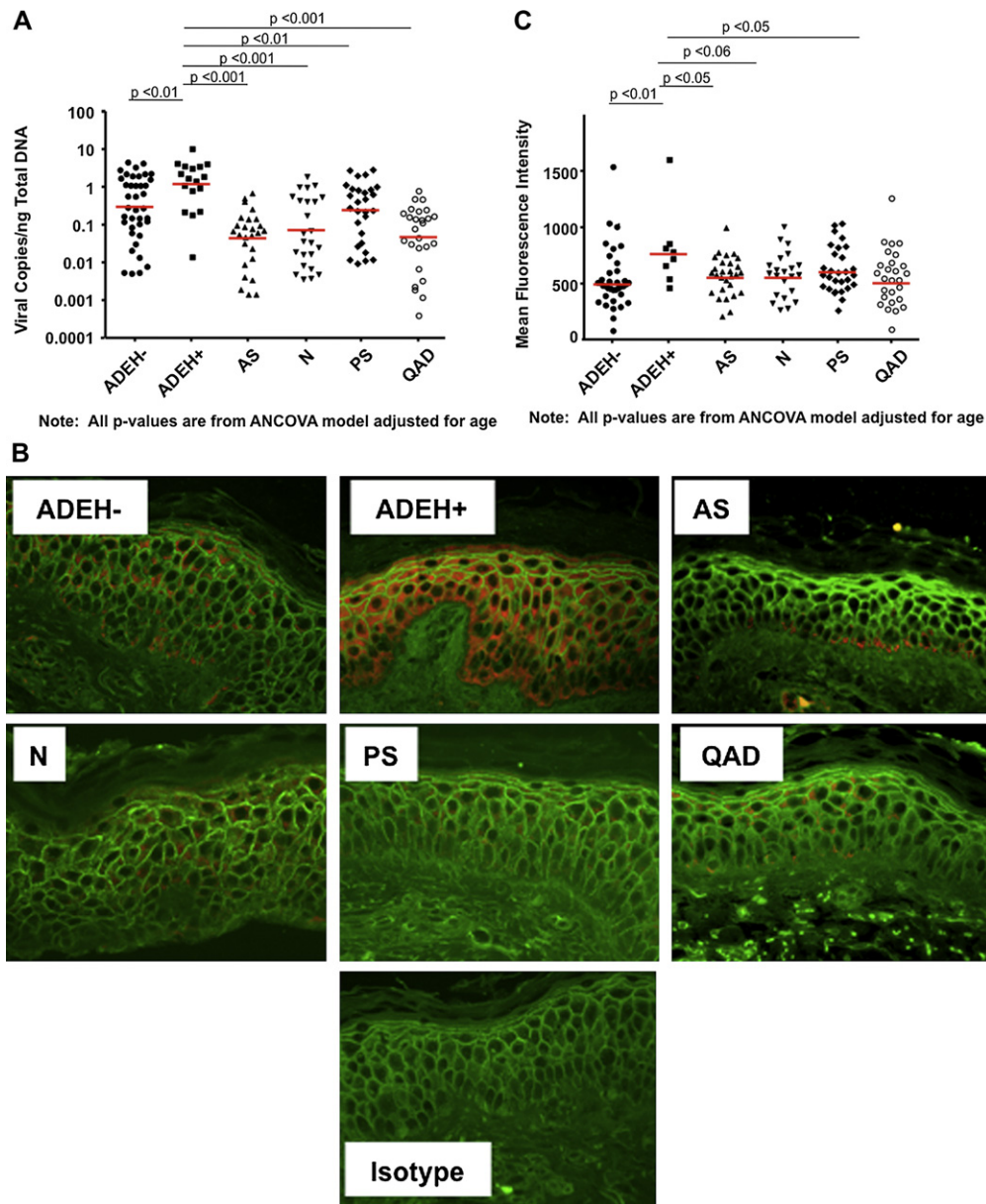


FIG 1. ADEH+ skin supports significantly greater VV replication. **A**, DNA was isolated from media-or VV-stimulated nonlesional skin and analyzed for VV gene expression by means of real-time RT-PCR. **B**, Immunofluorescent staining for A27L. **C**, MFI for VV expression in the basal keratinocytes of each biopsy specimen. AS, Asthmatic patients; N, healthy subjects; PS, patients with psoriasis; QAD, patients with quiescent AD. Significant differences: * $P < .05$, ** $P < .01$, and *** $P < .001$.

log-rank test; Fig 3, B). In a separate set of experiments, we further evaluated the primary lesions of mice on day 7 to determine whether STAT-6 overexpression potentiated VV pathogenesis. VV replication was significantly higher in the primary lesions of STAT-6VT mice (26.84 ± 20.67 ng of VV/ng of glyceraldehyde-3-phosphate dehydrogenase, $P < .05$) compared with that seen in wild-type mice (4.19 ± 2.69 ng of VV/ng of glyceraldehyde-3-phosphate dehydrogenase; Fig 3, C). Increased VV replication was confirmed by using immunofluorescent staining for A27L (Fig 3, D). The MFI for A27L was significantly greater in primary lesions of 6 STAT-6VT mice (277.0 ± 30.0 MFI, $P \leq .01$) compared with that seen in primary lesions from 6 wild-type mice (183.3 ± 13.4 MFI).

Association of *STAT6* variants with risk of ADEH+ status

IL-4 and IL-13 potentiate T_H2 responses by signaling through *STAT6*.^{19,20} To test for association between *STAT6* SNPs and ADEH+ status, we genotyped a total of 10 SNPs in a white patient sample. Two linkage disequilibrium (LD) blocks were identified, consisting of one approximately 13-kb block covering the *STAT6* gene and one approximately 1-kb block covering part of the promoter region (Fig 4, A). As seen in Table III, significant associations with ADEH+ status were observed for 3 intronic SNPs (rs3024975: OR, 2.14; 95% CI, 1.15-4.01, $P = .009$; rs841718: OR, 1.66; 95% CI, 1.14-2.41; $P = .006$; and rs167769: OR, 0.65; 95% CI, 0.43-0.98; $P = .027$) and one 3'-untranslated region

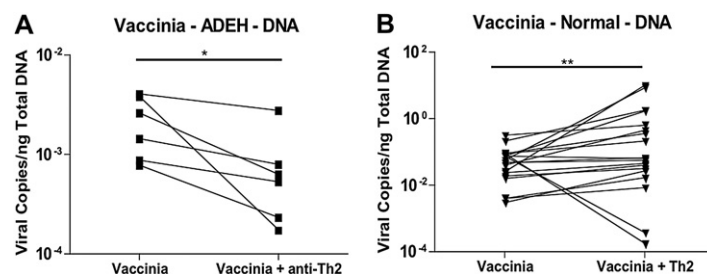


FIG 2. IL-4 and IL-13 modulate VV replication. VV expression in nonlesional skin from ADEH- (A; n = 17) and ADEH+ (B; n = 6) patients infected with VV after pretreatment with or without neutralizing antibodies to IL-4 and IL-13 is shown. Significant difference: * $P < .05$.

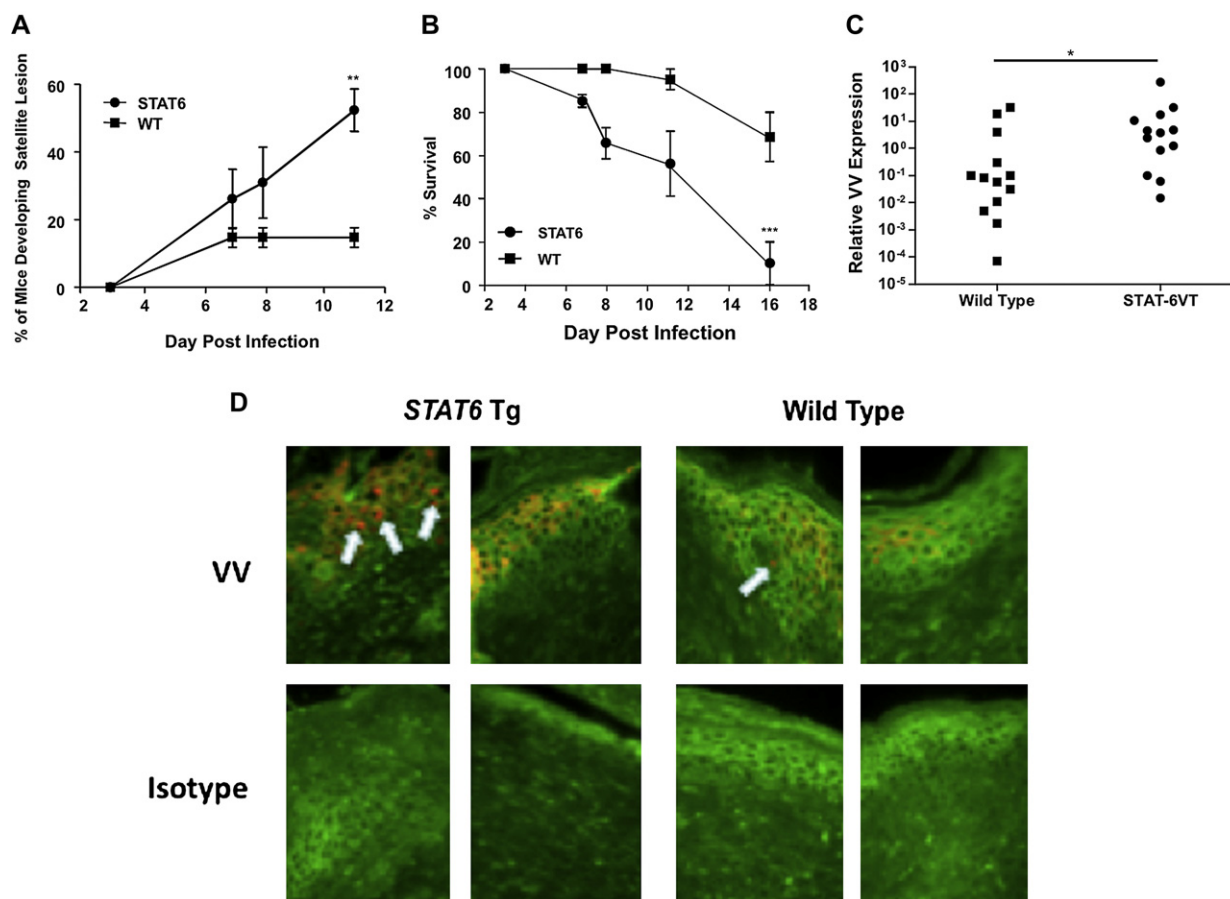


FIG 3. Constitutive *STAT6* expression predisposes mice to disseminated VV infection. Satellite lesions (A), mortality rate (B), VV gene expression (C), and VV protein (D) staining in wild-type (WT) and STAT-6VT mice after infection with 5×10^6 platelet-forming units of VV are shown. VV replication was visualized by staining for the surface protein A27L (red) and wheat germ agglutinin (green) to visualize the epidermis. Tg, Transgenic. Significant differences: * $P < .05$, ** $P < .01$, and *** $P < .001$.

SNP (rs703817; OR, 1.40; 95% CI, 0.92-2.15; $P = .029$). The associations were further enhanced by 2- to 5-marker haplotype analysis with omnibus P values from 2.47×10^{-5} to .037; Fig 4, B). Among these haplotype analyses, the strongest signal was observed for a 2-SNP haplotype (CT) comprised of SNP rs167769 and rs324013 spanning a region of less than 6.89 kb in the promoter region of *STAT6* (ADEH+ vs ADEH-, 24.9% vs 9.2%; $P = 5.17 \times 10^{-6}$; Table IV). A suggestive association was also observed for SNP rs324011 (OR, 0.70; 95% CI, 0.47-1.01; $P = .06$). In addition, we tested for association between

STAT6 SNPs and tIgE levels and Eczema Area and Severity Index scores, but no association was found for any of those tested SNPs. Similarly, no associations were observed between the *STAT6* variants and the risk of AD.

***STAT6* variants are associated with IFN- γ ELISPOT values**

In previous studies we have observed a reduced IFN- γ production in ADEH+ patients when compared with that seen

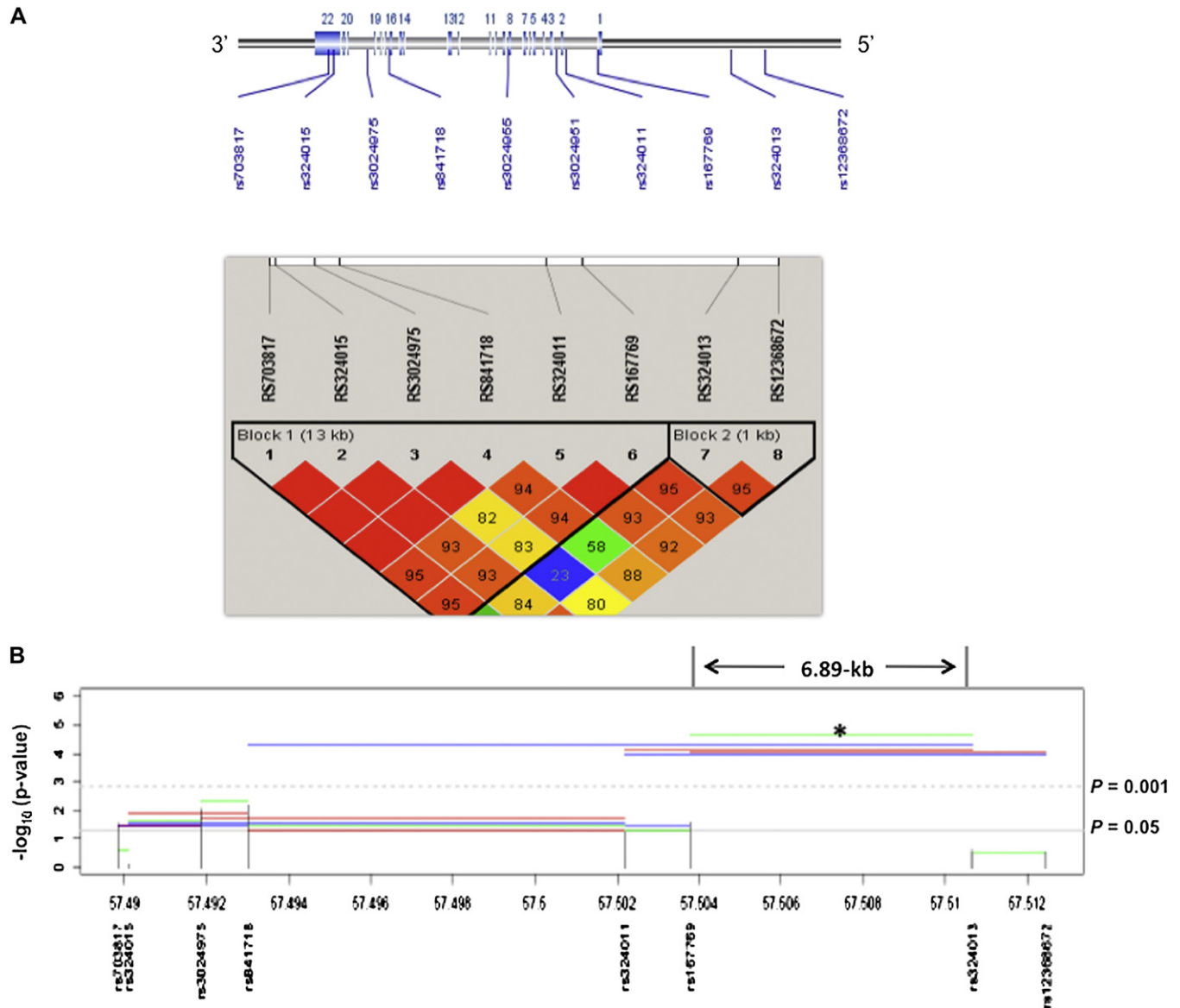


FIG 4. Analysis of *STAT6* polymorphisms in white patient samples. **A**, *STAT6* gene structure and distribution of the genotyped SNPs across the gene (22.6 kb) on chromosome 12q13 haplotype block structure of *STAT6* SNPs in white healthy control subjects ($n = 166$) is presented. The intensity of shading represents D' (a measure of LD) generated by using HAPLOVIEW software, with red (100) to green reflecting higher to lower D' values. Both rs3024951 and rs3024955 were excluded in the LD plot because of their low allele frequencies. **B**, Haplotype results showing omnibus P values constructed across sliding windows of sizes 2 to 5 for 8 common SNPs and ADEH+. Black vertical lines represent all individual SNP tests, and colored horizontal lines represent 2, 3, 4, and 5 haplotype tests. *See the detailed data in Table IV.

in ADEH⁻ patients and healthy control subjects.¹⁴ To explore the functional link of *STAT6* variants to disease, we tested whether patients carrying these SNPs have differential levels of IFN- γ . We found a significant association for SNP rs3024951 and IFN- γ levels ($P = .031$) when analyses were made for all these SNPs and IFN- γ levels in HSV-stimulated PBMCs from all subjects ($n = 64$). Moreover, when analyses were restricted to patients with AD ($n = 44$), the significance was further enhanced (TC vs TT, $P = .008$; Fig 5, A). In addition, we observed a significant association for the promoter SNP rs324013. Interestingly, the SNP rs324913 T allele, which has been shown to have the strongest association with an

increased risk of ADEH⁺ status as part of 2-marker haplotype showed lower levels of IFN- γ compared with others (log₁₀-transformed spot-forming units, CC vs CT vs TT, 2.31 vs 2.02 vs 1.86; $P = .025$; Fig 5, B).

DISCUSSION

Eczema vaccinatum is a severe, life-threatening adverse event that develops in a subset of patients with AD after smallpox vaccination. Our current study is the first to demonstrate that the skin of ADEH⁺ patients supports VV replication to a greater extent than the skin of ADEH⁻ patients and patients with

TABLE III. Associations between *STAT6* SNPs and ADEH

dbSNP	Position	Role	Risk allele	MAF		OR (95% CI)	P value
				ADEH+	ADEH–		
rs12368672	57512470	Promoter	G	0.321	0.376	0.78 (0.49-1.24)	.276
rs324013	55796928	Promoter	T	0.453	0.503	0.82 (0.56-1.19)	.285
rs167769	55790042	Intron	C	0.302	0.400	0.65 (0.43-0.98)	.027
rs324011	55788449	Intron	C	0.323	0.406	0.70 (0.47-1.01)	.060
rs841718	55779263	Intron (boundary)	A	0.505	0.381	1.66 (1.14-2.41)	.006
rs3024975	55778130	Intron	G	0.141	0.071	2.14 (1.15-4.02)	.009
rs324015	55776367	Downstream	C	0.261	0.252	1.05 (0.68-1.62)	.818
rs703817	57489828	Downstream	C	0.552	0.467	1.40 (0.92-2.15)	.029

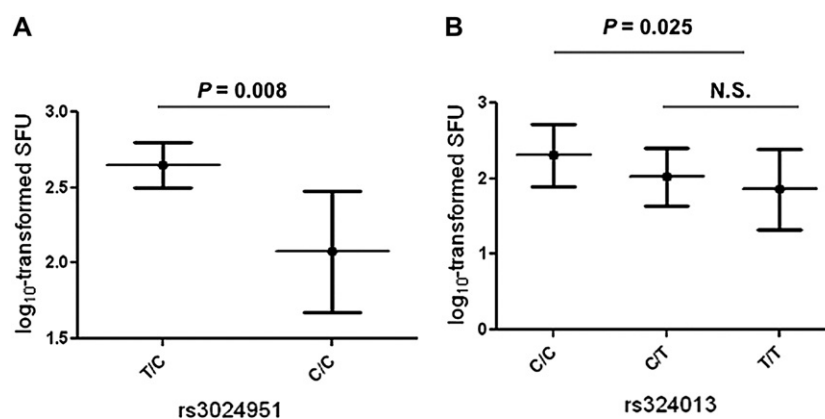
MAF, Minor allele frequency.

TABLE IV. Association of CT (rs167769 and rs324013) in *STAT6* and ADEH

Haplotype	rs167769	rs324013	MAF		OR (95% CI)	P value
			ADEH+	ADEH–		
1	C	C	0.45	0.51	0.77 (0.43-1.40)	.210
2	T	T	0.31	0.40	0.66 (0.31-1.24)	.038
3	C	T	0.25	0.09	3.33 (1.39-8.55)	5.17×10^{-6}

Omnibus *P* value = 2.47×10^{-5} .

MAF, Minor allele frequency.

**FIG 5.** Association of *STAT6* SNPs with IFN- γ production in HSV-stimulated PBMCs from patients with AD ($n = 44$) as determined by using the $\log_{10}$ -transformed mean spot-forming units (SFC)/ 10^6 cells. **A**, The association was observed for SNP rs3024951 (TC [$n = 7$] vs CC [$n = 32$]), $P = .008$. **B**, The association was observed for SNP rs324013 (CC [$n = 13$] vs CT [$n = 18$] + TT [$n = 8$], $P = .025$). N.S., Not significant.

quiescent AD, as well as non-AD patient groups, including those with asthma and psoriasis. Increased VV replication was confirmed by analyzing the number of VV DNA copies and immunofluorescent staining for A27L, a protein essential for transport of intracellular virus particles.²¹ We hypothesize that increased VV replication in skin explants can predict those patients who are at risk of a disseminated viral skin infection after smallpox vaccination.

Previously, we have shown that overexpression of IL-4 and IL-13 in AD skin supports increased VV replication in human keratinocytes.^{22,23} IL-4 and IL-13 have previously been shown to signal through *STAT6*,^{19,20} which binds to consensus target sequences and inhibits transcription of innate immune response genes.^{24,25} Specifically, we have demonstrated that IL-4 and IL-13 activate *STAT6* signaling, thereby inhibiting the

induction of antimicrobial peptides that have activity against VV.^{22,25} In the current study we demonstrate that neutralization of IL-4 and IL-13 in ADEH+ skin limits VV replication. It is unethical to test vaccination in patients with AD, and therefore we used transgenic mice as a surrogate to evaluate whether constitutive *STAT6* activation predisposes mice to disseminated VV skin infection. Transgenic mice overexpressing constitutively active *STAT6* in T cells are predisposed toward T_H2 responses and allergic inflammation, as previously described.^{13,26} These mice spontaneously have eczematous skin rashes, overexpress IL-4 and IL-13 in the skin, and exhibit increased serum IgE levels caused by increased T_H2 development *in vivo*.²⁷ It has previously been shown, by using knockout mice, that VV replication is impaired in IL-4-deficient mice,²⁸ which is consistent with the increased mortality seen in C57BL/6 mice infected with

an IL-4-expressing Ectromelia virus (mousepox).²⁹ In our experiments constitutive activation of STAT-6 increased morbidity and mortality in mice after VV infection. This was evidenced by increased satellite lesion development and decreased survival in STAT-6VT mice. Given the importance of STAT-6 in IL-4- and IL-13-mediated VV replication in AD skin,⁹ we sought further support for this molecule in susceptibility of disseminated viral skin infections among patients with AD by genotyping genetic polymorphisms encompassing the *STAT6* gene and testing for association with ADEH+ and associated phenotypes in white patients participating in the multicenter ADVN.¹⁰ Indeed, genetic linkage studies have previously identified the chromosomal region 12q13-24, where *STAT6* is located, to be linked with atopy-related diseases.³⁰ Genetic variants in *STAT6* have been associated with atopy,³¹ tIgE levels,^{13,32,33} and asthma^{13,34} in diverse populations. In particular, a polymorphism (rs324011) in intron 2 of *STAT6* that was associated with increased serum IgE levels was suggested to alter nuclear factor κ B binding, *STAT6* promoter activity, and mRNA expression.^{32,35} In this study in addition to those significant associations observed for 4 *STAT6* SNPs (rs3024975, rs841718, rs167769, rs703817) and ADEH+ status, we provided suggestive evidence of an association between ADEH and SNP rs324011. This marker was in strong LD with the strongest SNP with ADEH- association (rs841718) within the same LD block ($D' = 0.94$; Fig 3, B). The association with ADEH was further enhanced by haplotype analysis, in which we observed that haplotype CT (a 2-marker haplotype, rs167769 C-rs324013T) across a region of 6.87 kb was significantly more common in ADEH+ patients compared with ADEH- patients (24.9% vs 9.2%, $P = 5.17 \times 10^{-6}$). Interestingly, this haplotype encompasses the major functional region in the promoter of *STAT6*. We contend that either this haplotype or an untyped genetic variant or variants within this locus might confer susceptibility to an increased risk of ADEH and have a functional effect on disease. Although *STAT6* SNPs have been repeatedly associated with tIgE levels,^{32,33,36} none of those identified SNPs for ADEH+ status in this study were associated with this trait (data not shown), suggesting that the genetic associations are specific to disease risk. To test for the possibility that the associations observed between *STAT6* SNPs and ADEH were for manifestation of an HSV exacerbation in an AD population rather than the disease markers, we investigated the association between HSV infection and the associated SNPs among healthy control subjects, none of which showed association with HSV infection (data not shown), suggesting that the associations with ADEH were not confounded by HSV infection.

Although the genetic association studies herein were intended to support the VV expression studies and transgenic murine studies, a shortcoming of these analyses is a lack of replication in an independent sample. In addition, to explore whether abnormalities in STAT6 function coexist with a filaggrin (*FLG*)-dependent barrier defect, we performed a gene-gene interaction analysis and found that there was no increased association for the combined *STAT6* SNPs and *FLG* mutations compared with SNPs analyzed individually. The results suggested that the associations observed for *STAT6* SNPs might be independent of *FLG* mutations, but this needs to be replicated in a relatively large population with a comprehensive coverage of *STAT6*.

One of the major strengths in this study is that we observed significant associations between variants in *STAT6* and IFN- γ production in HSV-stimulated PBMCs from patients with AD. Among all subjects, both SNP rs3024951 and SNP rs324013 showed differential IFN- γ production in response to HSV exposure. Although the precise mechanism is not clear, our findings suggest that these *STAT6* SNPs might be involved in the regulation of IFN- γ production. Indeed, the SNP rs324013 T allele, one part of the 2-marker haplotype CT (rs167769 C-rs324013T), showed the strongest association with an increased risk of ADEH+ status but reduced levels of IFN- γ production, suggesting that this SNP or SNPs within the region covering the haplotype might play a role in mediating HSV-induced IFN- γ production and subsequently lead to the development of ADEH+. As a result, it will be essential to investigate additional SNPs in and around the identified region (approximately 6.87 kb) in the future.

We recognize that this study has several limitations. In addition to the absence of a replication population, the sample available for these studies is relatively small. However, ADEH is a rare disease (approximately 3%), and the sample used for this study reflects intense recruitment efforts after nearly 5 years from multiple medical centers. As demonstrated in the Methods section, we were in fact sufficiently powered to detect a true association given the frequency of the SNPs associated with ADEH+ status (ie, >10% to 20%). Second, our observations in these initial studies relying heavily on a tagging SNP approach are the basis for further studies. As a next step, we aim to identify the causal *STAT6* variant or variants by using an independent replication with sufficient sample size, deep resequencing of the candidate region elucidated herein, and ultimately determining the functional relevance to risk of ADEH+ status.

In the current study we demonstrate that skin from ADEH+ patients supports VV replication. Using STAT-6VT transgenic mice, we demonstrate a functional correlation between *STAT6* activity in T cells and disseminated VV infection. Furthermore, we provide evidence of an association between *STAT6* SNPs and the risk of ADEH and IFN- γ production in a multicenter case-control study. Although the mechanisms remain unclear, our findings suggest that *STAT6* SNPs might be involved in the regulation of IFN- γ production and also serve as important genetic markers in determining those patients with AD with greater susceptibility to disseminated VV infection after smallpox vaccination. This is the first study to implicate *STAT6* as a potential candidate gene for ADEH. However, replication in an independent population with sufficient sample size, evidence of association between *STAT6* SNPs and skin IL-4 and IL-13 expression, and evidence of functional relevance to disease are clearly needed in future studies. Additionally, given that *FLG* in our previous studies has been shown to have a significant effect on ADEH, further analysis on an interaction between *FLG* and *STAT6* is essential. Taken together, our data suggest that the *STAT6* gene increases viral replication in the skin of patients with ADEH. Further genetic association studies and functional investigations are warranted.

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Key messages

- Increased IL-4 and IL-13 expression enhances viral replication in atopic skin.
- Activation of the *STAT6* gene enhances viral replication in patients with AD.
- Polymorphisms in the *STAT6* gene are associated with propensity for EH.

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